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Circulating Total Bilirubin and Future Risk of Hypertension in the General Population: The Prevention of Renal and Vascular End-Stage Disease (PREVEND) Prospective Study and a Mendelian Randomization Approach

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***Background*—**Circulating total bilirubin is known to be inversely and independently associated with future risk of cardiovascular disease. However, the relationship of circulating total bilirubin with incident hypertension is uncertain. We aimed to assess the association of total bilirubin with future hypertension risk and supplemented this with a Mendelian randomization approach to investigate any causal relevance to the association.

***Methods and Results*—**Plasma total bilirubin levels were measured at baseline in the PREVEND (Prevention of Renal and Vascular End-Stage Disease) prospective study of 3989 men and women without hypertension. Hazard ratios (95% confidence intervals) of total bilirubin with incident hypertension were assessed. New-onset hypertension was recorded in 1206 participants during a median follow-up of 10.7 years. Baseline total bilirubin was approximately log-linearly associated with hypertension risk. Age- and sex-adjusted hazard ratio for hypertension per 1-SD increase in $\log_e$ total bilirubin was 0.86 (0.81–0.92; $P<0.001$), which was attenuated to 0.94 (0.88–0.99; $P=0.040$) after further adjustment for established risk factors and other potential confounders. The association was marginally significant on further adjustment for high-sensitivity C-reactive protein (0.94; 0.88–1.00; $P=0.067$). A genetic variant at the UGT1A1*28 locus consistently shown to be strongly associated with circulating bilirubin levels—rs6742078—was not significantly associated with blood pressure or hypertension ($P>0.05$ for all), arguing against a strong causal association of circulating bilirubin with blood pressure.

***Conclusions*—**The weak and inverse association of circulating total bilirubin with future hypertension risk may be driven by biases such as unmeasured confounding and/or reverse causation. Further evaluation is warranted. **(*J Am Heart Assoc*. 2017;6: e006503. DOI: 10.1161/JAHA.117.006503.)**

Key Words: bilirubin • cohort study • hypertension • Mendelian randomization • risk factor

Circulating total bilirubin has been consistently shown to be inversely and independently associated with cardiovascular disease (CVD) risk.[1] Plausible mechanisms by which higher total bilirubin contributes to reduced CVD risk have been attributed to its antioxidant,[2,3] anti-inflammatory,[4] and antiatherogenic properties.[5] Given the graded, inverse, and independent association between total bilirubin levels and CVD risk, there have been suggestions of a causal relationship. However, findings from 2 Mendelian randomization (MR) studies have not provided strong evidence for a causal association between total bilirubin levels and coronary heart disease risk.[6,7] Hypertension, which is a leading risk factor

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Accompanying Tables S1 through S3 and Figures S1, S2 are available at http://jaha.ahajournals.org/content/6/11/e006503/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- In a population-based, prospective study of white men and women without a history of hypertension and pre-existing apparent disease at baseline, increasing levels of circulating total bilirubin was associated with a reduced risk of future hypertension, which was consistent with a dose-response relationship.
- Findings from a Mendelian randomization approach provided weak evidence against a strong causal association of circulating bilirubin with blood pressure.

What Are the Clinical Implications?

- Lifestyle interventions and pharmacological agents that cause safe elevations in circulating levels of bilirubin may represent a novel therapeutic target for the prevention of hypertension and, consequently, cardiovascular disease; however, further evaluation is needed.

for the global burden of disease, is a key intermediate modifiable phenotype for CVD development[8] and is included in the standard cardiovascular risk assessment panel.[9] Major risk factors for hypertension include physical inactivity, obesity, and excess alcohol intake.[10,11] Given the close link between CVD and hypertension and the fact that they share common antecedent risk factors, there is emerging evidence that total bilirubin might also be linked to the development of hypertension. Indeed, a number of studies conducted in animal models suggest that bilirubin might reduce blood pressure through decreases in vascular oxidative stress.[12,13] A number of epidemiological observational studies have also suggested inverse associations. However, uncertainties remain about the nature and magnitude of the prospective association between total bilirubin and high blood pressure or hypertension, given that the majority of these limited earlier reports were based on cross-sectional designs,[14] were insufficiently powerful to address aspects of the association,[15,16] were based on younger populations,[17] or were conducted in populations with pre-existing disease.[15] In a recent analysis of data from the National Health and Nutrition Examination Surveys 1999–2012, which was based on a random sample of over 31 000 individuals, the researchers reported an inverse association between serum bilirubin and hypertension.[14] Though the analysis was robust, the findings were limited by the cross-sectional study design. With the ongoing debate on the potential value of total bilirubin levels in prevention and control of hypertension as well as coronary heart disease,[14,18–20] it will be clinically useful if circulating total bilirubin is shown to contribute to the development of future hypertension. In this context, our aim was to characterize and quantify more reliably the nature and magnitude of the prospective association between total bilirubin and the risk of future hypertension in the general population; by utilizing a large population-based sample of 3989 participants from the well-established PREVEND (Prevention of Renal and Vascular End-Stage Disease) study, who were free of hypertension and pre-existing apparent disease at baseline. Furthermore, we evaluated whether there might be a causal relation between total bilirubin and the development of hypertension, by querying the associations of systolic blood pressure (SBP), diastolic blood pressure (DBP), and hypertension with a common single-nucleotide variant (SNV) found at the UGT1A1*28 locus—rs6742078—using published genome-wide association studies. Because of the strong link between the rs6742078 SNV and bilirubin,[21,22] the use of this variant to assess the causal association between bilirubin and blood pressure is an informative application of the MR approach.

Materials and Methods

This report was conducted according to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for reporting observational studies in epidemiology (Table S1).[23]

Study Population

This study was part of the ongoing PREVEND study, a large-scale, observational, general population cohort study based in The Netherlands and which began in 1997. The PREVEND study was designed to investigate the predictive value of urinary albumin excretion and its relationship to renal and CVD progression. Details of the study design and recruitment have been described in previous reports.[24,25] Briefly, 8592 inhabitants aged 28 to 75 years were recruited from the city of Groningen in The Netherlands. Baseline measurements were performed between 1997 and 1998. For this analysis, we used data of participants who did not have CVD, hypertension, renal disease, or malignancy at baseline, which left a cohort of 3989 participants with nonmissing information on total bilirubin levels, relevant covariates, and incident hypertension. The PREVEND study was approved by the local medical ethics committee in accord with the Declaration of Helsinki. All participants provided written informed consent.

For the genetic association study, we utilized publicly available data from the International Consortium of Blood Pressure and the BPExome consortia, which have both been described in detail elsewhere.[26,27] Briefly, the International Consortium of Blood Pressure involves a meta-analysis of

genome-wide association studies data evaluating the associations between 2.5 million genotyped or imputed single-nucleotide polymorphisms and SBP and DBP in 69 395 individuals of European ancestry from 29 studies. The BPExome consortium is also a meta-analysis of genome-wide association studies data from 51 studies comprising 192 763 individuals, which assessed the associations of 242 296 SNVs with DBP, SBP, pulse pressure, and hypertension. The rs6742078 SNV was a suitable instrumental variable for the present analyses, given its robust specificity for serum total bilirubin levels (explaining up to 45% of the variation in circulating serum bilirubin levels[22]) and its use in previous studies to assess the causal relevance of total bilirubin to several disease outcomes.[6,7,21]

Risk-Factor Assessment

Participants completed 2 outpatient visits to assess baseline data on demographics, anthropometric measurements, cardiovascular and renal history, and use of medication. Furthermore, information on medication use was complemented with data from all community pharmacies in the city of Groningen, which covers complete information on drug use in 95% of PREVEND participants. Blood pressure values were recorded as the mean of the last 2 readings of both visits, because this provides the values after stabilization of blood pressure. Blood pressure was measured at the right arm, in the supine position, every minute for 10 and 8 minutes, respectively, with an automatic device (Dinamap XL Model 9300; Johnson-Johnson Medical, Tampa, FL). After an overnight fast and 15 minutes of rest, venous blood was obtained from participants. Plasma samples were prepared by centrifugation at 4°C. Plasma total bilirubin was measured by a colorimetric assay (2,4-dicholoraniline reaction; Merck MEGA, Darmstadt, Germany), with the detection limit being 1.0 mmol/L. Interassay coefficients of variation were 3.8% and 2.9% in the lower normal and higher normal range, respectively. Glucose, total cholesterol, fasting insulin, and hsCRP (high-sensitivity C-reactive protein) were measured using standard laboratory protocols, which have been previously described.[28–30] Urinary albumin excretion was estimated as the mean of two 24-hour urine collections, and the concentration was determined by nephelometry (BNII; Dade Behring Diagnostics, Marburg, Germany). Estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation.[31]

End Point Ascertainment

The primary outcome for this study was first-onset hypertension. Incident hypertension was defined as SBP of ≥140 mm Hg, a DBP of ≥90 mm Hg, or the use of antihypertensive medication, in accord with recommendations from the Seventh Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure.[32]

Statistical Analysis

Skewed variables (eg, total bilirubin and hsCRP) were natural log-transformed to achieve approximately normal distributions. Descriptive analyses were preformed to summarize baseline characteristics of participants. We assessed cross-sectional associations of total bilirubin levels with risk markers for hypertension by calculating partial correlation coefficients adjusted for age and sex. Cox proportional hazards models were used to assess the association between total bilirubin and incident hypertension risk after confirmation of no major departure from the proportionality of hazards assumptions using Schoenfeld residuals.[33] To characterize the shape of the association between total bilirubin and hypertension risk, hazard ratios estimated within quartiles of baseline total bilirubin levels relative to the bottom quartile were plotted against mean $\log_e$ total bilirubin levels in each quartile using floating absolute risks,[34] details of which have been described previously.[35] Subsidiary analyses involved fitting multivariate-adjusted fractional polynomial models. Total bilirubin was modeled as both continuous (per 1-SD higher $\log_e$ total bilirubin levels) and categorical (quartiles defined according to the baseline distribution of total bilirubin level) variables. The SD of baseline $\log_e$ total bilirubin level was 0.43 (equivalent to 1.5-fold higher circulating total bilirubin level, as $e^{0.43}=1.54$). Hazard ratios were progressively adjusted for (1) age and sex; (2) other established risk factors for hypertension (smoking status, history of diabetes mellitus, SBP, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate); (3) other potential confounders (urinary albumin excretion and homeostasis model assessment of insulin resistance); and (4) hsCRP. Selection of these confounders were based on their previously established role as risk factors for hypertension, evidence from previous research, or their potential as confounders based on known associations with the outcome of hypertension and observed associations with plasma total bilirubin using the available data. We used formal tests of interaction tests to assess statistical evidence of effect modification by individual characteristics, such as age, sex, and other risk markers for hypertension. To avoid potential bias attributed to reverse causation, we carried out sensitivity analyses that excluded participants with a history of diabetes mellitus at baseline, the first 2 years of follow-up, or participants on regular statin medication, or participants

with potential Gilbert's syndrome.[1] We also utilized the use of complex survey design analyses,[36] taking into account that the PREVEND cohort is oversampled for subjects with higher albuminuria levels, which enables the results to be extrapolated to the general population. All statistical analyses were conducted using Stata software (version 14; StataCorp LP, College Station, TX). The associations of rs6742078 were queried with SBP and DBP using data from the International Consortium of Blood Pressure and with SBP, DBP, and hypertension using data from the BPExome consortium.[37]

Results

Baseline Characteristics and Correlates of Total Bilirubin

Mean age of participants at study entry was 45 (SD, 11) years and 55% were women. Mean (SD) of $\log_e$ total bilirubin level was 1.94 (0.43) μmol/L. Figure S1 shows a histogram representing the frequency distribution of total bilirubin levels in the study sample. Baseline descriptive characteristics of the participants are shown in Table 1. Except for parental history of hypertension, there were

Table 1. Baseline Participant Characteristics Overall and According to the Development of Incident Hypertension

	Overall (N=3989) Mean (SD) or Median (IQR) or n (%)	Without Incident Hypertension (N=2783) Mean (SD) or Median (IQR) or n (%)	With Incident Hypertension (N=1206) Mean (SD) or Median (IQR) or n (%)	*P* Value*
Total bilirubin, μmol/L†	7 (5–9)	7 (5–9)	7 (5–9)	<0.001
Questionnaire				
Males‡	1790 (44.9)	1188 (42.7)	602 (49.9)	<0.001
Age at survey, y§	45 (11)	43 (10)	50 (10)	<0.0001
History of diabetes mellitus‡	19 (0.5)	9 (0.3)	10 (0.8)	0.033
Smoking‡				
Current	1365 (34.2)	942 (33.9)	423 (35.1)	
Former	1351 (33.9)	914 (32.8)	437 (36.2)	0.012
Never	1273 (31.9)	927 (33.3)	346 (28.7)	
Alcohol consumers‡	3121 (78.2)	2217 (79.7)	904 (75.0)	0.001
Parental history of hypertension‡	1185 (29.7)	807 (29.0)	378 (31.3)	0.136
Physical measurements				
BMI, kg/m^2§	25 (4)	25 (3)	26 (4)	<0.0001
WHR§	0.86 (0.09)	0.84 (0.08)	0.88 (0.09)	<0.0001
SBP, mm Hg§	119 (11)	116 (10)	125 (10)	<0.0001
DBP, mm Hg§	70 (7)	68 (7)	74 (7)	<0.0001
Lipid, metabolic, inflammatory, and renal markers				
Total cholesterol, mmol/L§	5.48 (1.11)	5.35 (1.07)	5.77 (1.14)	<0.0001
Glucose, mmol/L§	4.63 (0.87)	4.55 (0.71)	4.82 (1.13)	<0.0001
Fasting insulin, units/mL†	7.1 (5.1–10.2)	6.8 (4.9–9.6)	7.8 (5.5–11.6)	<0.0001
HOMA-IR†	1.43 (0.99–2.12)	1.36 (0.96–1.94)	1.65 (1.10–2.50)	<0.0001
hsCRP, mg/L†	0.95 (0.43–2.30)	0.85 (0.38–2.00)	1.29 (0.58–2.87)	<0.0001
eGFR, mL/min per 1.73 m^2§	92.3 (14.0)	93.7 (13.5)	88.9 (14.3)	<0.0001
UAE, mg/24 h†	8.04 (5.86–12.45)	7.61 (5.72–11.48)	9.18 (6.38–15.55)	<0.0001

BMI indicates body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; IQR, interquartile range; SBP, systolic blood pressure; UAE, urinary albumin excretion; WHR, waist-to-hip ratio.

*Utilized a 2-sample *t* tests for a difference in means for continuous variables and a chi-square test for categorical variables.

†Reported as median (IQR).

‡Reported as n (%).

§Reported as mean (SD).

significant differences in baseline clinically relevant subgroups and levels of risk markers between participants who did and did not develop hypertension during follow-up. There were weak and inverse correlations of $\log_e$ total bilirubin levels with physical measures (body mass index, waist-to-hip ratio, and blood pressure), as well as with cholesterol and metabolic markers. There was a modest inverse correlation with $\log_e$ hsCRP (r=−0.25). Baseline total bilirubin levels were higher by 25% in men compared with women. Levels were lower by 13% in the combined group of current and former smokers compared with noncurrent smokers (Table 2).

Total Bilirubin Levels and Risk of Incident Hypertension

During a median (interquartile) follow-up of 10.7 (5.5–11.6) years, 1206 incident hypertension cases (incidence rate of 34.3 per 1000 person-years at risk; 95% confidence interval [CI], 32.4–36.3) were recorded. Total bilirubin was approximately log-linearly associated with hypertension risk in analyses adjusted for established hypertension risk factors (smoking status, history of diabetes mellitus, SBP, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate; Figure 1). A linear shape was also suggested on fitting a fractional polynomial model (Figure S2). In age- and sex-adjusted analysis, the hazard ratio for hypertension per 1-SD change in $\log_e$ total bilirubin was 0.86 (95% CI, 0.81–0.92; P<0.001), which was attenuated to 0.94 (95% CI, 0.88–0.99; P=0.035) after further adjusting for several risk factors for hypertension. The results remained consistent on further adjustment for urinary albumin excretion and homeostasis model assessment of insulin resistance 0.94 (95% CI, 0.88–0.99; P=0.040). The association did not reach formal significance after additional adjustment for hsCRP 0.94 (95% CI, 0.88–1.00; P=0.067; Table 3). However, in an age- and sex-only–adjusted analysis, the initial association 0.86 (95% CI, 0.81–0.92; P<0.001) was only minimally attenuated after single additional adjustment for hsCRP 0.90 (95% CI, 0.85–0.96; P=0.001). In analyses that compared the top versus bottom quartile of total bilirubin, the inverse associations between total bilirubin and incident hypertension were maintained (Table 3). In sensitivity analyses, the hazard ratios remained similar on exclusion of the first 2 years of follow-up, people with diabetes mellitus at baseline, people on cholesterol lowering medication, or people with potential Gilbert's syndrome (Table S2). The association between total bilirubin and incident hypertension was not statistically significantly modified by several clinically relevant characteristics (Figure 2). The association between total bilirubin and hypertension risk remained consistent similar when design-based Cox regression analysis was used (Table S3).

Evidence From Genome-Wide Association Studies

In the International Consortium of Blood Pressure, the associations of rs6742078 with blood pressure were not statistically significant; 0.187 mm Hg per additional copy of the T allele (SE, 0.103; P=0.06) for SBP and 0.122 (SE, 0.066; P=0.07) for DBP. Similarly, in the BPExome consortium, associations were nonstatistically significant; P=0.24 for SBP, P=0.85 for DBP, and P=0.97 for hypertension. These results provide evidence against a strong causal role of long-term elevated levels of bilirubin in decreasing blood pressure.

Discussion

Key Findings

In this population-based study comprising white men and women without a history of hypertension and pre-existing disease at baseline, we have shown that total bilirubin is inversely associated with the future risk of hypertension in an approximately log-linear fashion. The association was independent of several established risk factors for hypertension and other potential confounders. The association was marginally significant on further adjustment for hsCRP; however, the association was only minimally attenuated after single adjustment for hsCRP in an analysis that was initially only adjusted for age and sex. The inverse association between total bilirubin and incident hypertension was not modified by several clinically relevant characteristics and remained consistent in several sensitivity analyses. Furthermore, utilizing large-scale genetic data, the rs6742078 SNV had small effects on blood pressure, but lacked statistical significance. The current results argue against a strong causal role of circulating bilirubin in the etiology of blood pressure reduction, but cannot rule out a weak causal effect.

Comparison With Previous Studies

A number of epidemiological studies have suggested an inverse association between total bilirubin and hypertension or blood pressure; but there were several limitations of these previous reports and which included utilization of cross-sectional designs, small sample sizes, or use of selected populations.[15,16,20,38] Chin et al, in their analysis of a cohort of 1208 normotensive Korean men and women, showed serum bilirubin to be associated with lower incidence of hypertension.[16] However, this analysis was somewhat limited by the relatively low event rate in the exposed group, the staggered follow-up evaluations, and the sampling frame,

Table 2. Cross-Sectional Correlates of Total Bilirubin

	Partial Correlation *r* (95% CI)*	Percentage Difference (95% CI) in Total Bilirubin Levels Per 1 SD Higher or Compared With Reference Category of Correlate†
Total bilirubin, μmol/L	…	…
Sex		
Female	…	Ref
Male	…	25% (21, 28)‖
Questionnaire		
Age at survey, y	−0.05 (−0.08, −0.01)‡	−2% (−3, −1)‡
History of diabetes mellitus		
No	…	Ref
Yes	…	−8% (−23, 12)
Smoking status		
Nonsmokers	…	Ref
Current and former smokers	…	−13% (−16, −11)‖
Alcohol consumption		
Nonconsumers	…	Ref
Current consumers	…	6% (3, 9)§
Parental history of hypertension		
No	…	Ref
Yes	…	−1% (−4, 2)
Physical measurements		
BMI, kg/m^2	−0.17 (−0.20, −0.14)‖	−7% (−8, −6)‖
WHR	−0.14 (−0.17, −0.10)‡	−7% (−9, −6)‖
SBP, mm Hg	−0.05 (−0.08, −0.02)‡	−2% (−4, −1)§
DBP, mm Hg	−0.09 (−0.12, −0.06)	−4% (−5, −3)‖
Lipid, metabolic, inflammatory, and renal markers		
Total cholesterol, mmol/L	−0.15 (−0.18, −0.12)‖	−7% (−8, −5)‖
Glucose, mmol/L	−0.09 (−0.12, −0.06)§	−4% (−5, −3)‖
Fasting insulin, units/mL	−0.21 (−0.24, −0.18)‖	−8% (−9, −7)‖
HOMA-IR	−0.21 (−0.24, −0.18)‖	−8% (−10, −7)‖
hsCRP, mg/L	−0.25 (−0.27, −0.22)‖	−10% (−11, −9)‖
eGFR, mL/min per 1.73 m^2	−0.01 (−0.04, 0.02)	−1% (−2, 1)
UAE, mg/24 h	−0.04 (−0.07, −0.01)	−1% (−2, 1)

BMI indicates body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; Ref, reference; SBP, systolic blood pressure; UAE, urinary albumin excretion; WHR, waist-to-hip ratio.

*Partial correlation coefficients between $\log_e$ total bilirubin and the row variables.

†Percentage change in total bilirubin levels per 1-SD increase in the row variable (or for categorical variables, the percentage difference in mean total bilirubin levels for the category vs the reference) adjusted for age and sex.

Asterisks indicate the level of statistical significance: ‡$P<0.05$; §$P<0.01$; ‖$P<0.001$.

which was not representative of the general population. In an elegant analysis of National Health and Nutrition Examination Surveys 1999–2012, Wang and Bautista robustly demonstrated that serum bilirubin was inversely associated with SBP and hypertension[14]; however, the main limitation of this study was its cross-sectional design, which precluded the ability to assess the temporal relationship between bilirubin and risk of hypertension and minimize reverse causation bias. To our

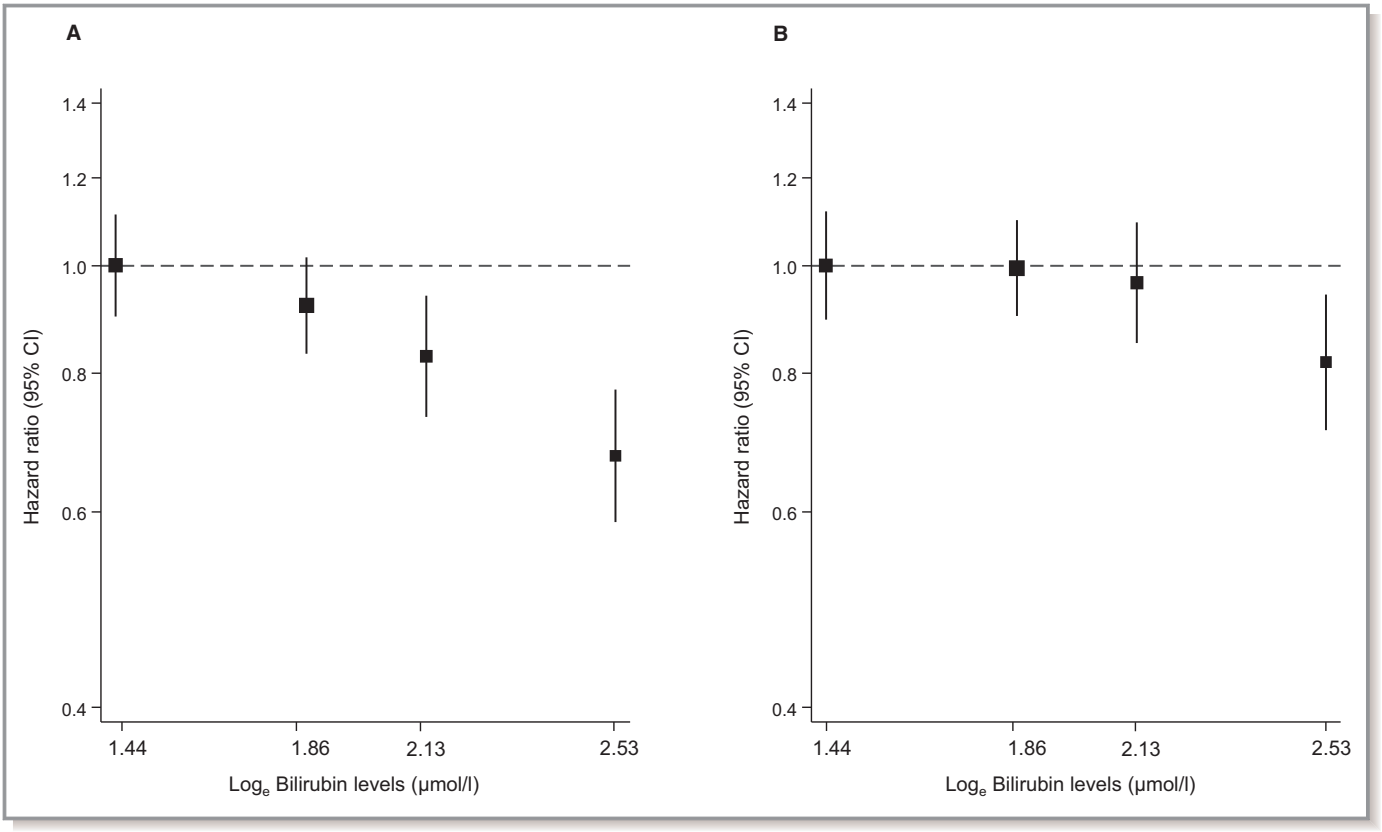

Figure 1. Hazard ratios for incident hypertension, by baseline levels of total bilirubin using floating absolute risks. A, Hazard ratios were adjusted for age and sex; (B) adjustment in A plus smoking status, history of diabetes mellitus, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation). CI indicates confidence interval.

knowledge, our study is the first to assess the long-term prospective association between total bilirubin and risk of hypertension in a general white population. We demonstrated a modest effect of total bilirubin on hypertension risk, a finding that was also demonstrated in the National Health and Nutrition Examination Surveys 1999–2012 analysis. In a recent study, which evaluated the association between bilirubin and several cardiovascular risk factors, the researchers demonstrated a lack of an effect of bilirubin on blood pressure in both observational and MR analyses.[22] Whether baseline circulating total bilirubin has an inverse association with future hypertension risk may need to be confirmed in other large-scale, prospective cohort studies, given the limited evidence.

Table 3. Association of Baseline Total Bilirubin Levels With Incident Hypertension

Total Bilirubin Level, μmol/L	Events/Total	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Per 1-SD increase	1206/3989	0.86 (0.81–0.92)	<0.001	0.94 (0.88–0.99)	0.035	0.94 (0.88–0.99)	0.040	0.94 (0.88–1.00)	0.067
Q1 (0.95–5)	367/1134	Ref		Ref		Ref			
Q2 (6–7)	381/1184	0.92 (0.80–1.06)	0.267	1.00 (0.86–1.16)	0.978	0.99 (0.86–1.15)	0.924	1.00 (0.86–1.16)	0.992
Q3 (8–9)	243/826	0.83 (0.70–0.98)	0.026	0.97 (0.82–1.14)	0.700	0.97 (0.82–1.15)	0.736	0.98 (0.83–1.16)	0.834
Q4 (≥10)	215/845	0.68 (0.57–0.81)	<0.001	0.82 (0.68–0.98)	0.033	0.82 (0.68–0.99)	0.035	0.83 (0.69–1.00)	0.055

Model 1: Age and sex. Model 2: Model 1 plus smoking status, history of diabetes mellitus, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation). Model 3: Model 2 plus log$_e$ urinary albumin excretion and log$_e$ homeostasis model assessment of insulin resistance. Model 4: Model 3 plus log$_e$ high-sensitivity C-reactive protein. CI indicates confidence interval; HR, hazard ratio; Q, quintile.

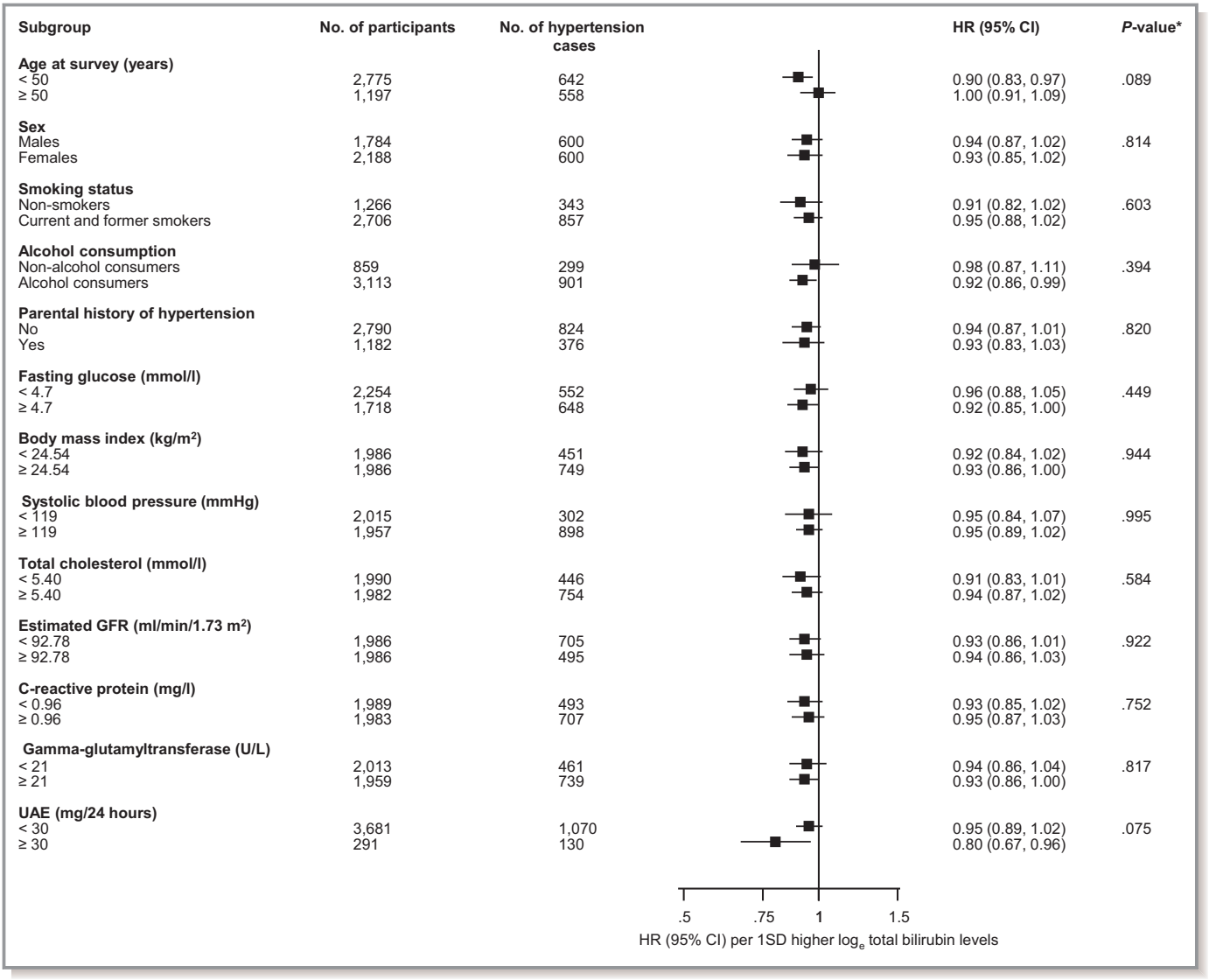

Figure 2. Hazard ratios for total bilirubin and hypertension risk by several participant level characteristics. Ratios were adjusted for age, sex, smoking status, history of diabetes mellitus, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (GFR; as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); CI indicates confidence interval (bars); HR, hazard ratio; UAE, urine albumin excretion. **P* value for interaction; cutoffs used for fasting glucose, body mass index, systolic blood pressure, total cholesterol, estimated GFR, and C-reactive protein are median values.

Potential Explanations for Findings

Bilirubin, iron, and carbon monoxide (CO) constitute the 3 metabolites of heme degradation by heme oxygenase (HO). These degradation products of the HO reaction have been suggested to regulate important functions in cells.[39] Bilirubin has been suggested to contribute to reduced CVD risk mainly through its antioxidant actions[2,3] and anti-inflammatory effects.[4] Like CVD, oxidative stress mechanisms are involved in the pathophysiology of hypertension.[40,41] Vascular reactive oxygen species, which are generated during oxidative reactions, are known to be important contributors to the development of hypertension,[42] because they cause impairment of mechanisms that modulate arterial blood pressure.[43,44] Increased production of reactive oxygen species in the renal medulla is primarily responsible for angiotensin-II–dependent hypertension.[45] Given this, it has been postulated that the protective effect of bilirubin on elevated blood pressure may be through its potent antioxidant effects.[46] It has been suggested that the primary role of bilirubin in the whole process is its inhibition of nicotinamide adenine dinucleotide phosphate oxidase, which is the main enzyme responsible for the generation of vascular reactive oxygen species.[47,48] Bilirubin

also inhibits protein kinase C activity[49] and scavenges the superoxide anions in vascular cells, which inhibits the pressor actions of angiotensin II.[13] In addition to its antioxidant effects, bilirubin exhibits anti-inflammatory effects through its anticomplement actions.[50] Given that inflammation has been implicated in the development of hypertension, circulating bilirubin might be involved in the modulation of blood pressure through its anti-inflammatory effects.[51] Finally, low serum bilirubin levels have been shown to be associated with impaired flow-mediated vasodilation,[52] a measure of endothelial dysfunction; which has been shown to precede the development of hypertension.[53] There is also a possibility that the blood-pressure–lowering effect of bilirubin could be partly attributed to CO, mostly known as a toxic gas if the exposure is high. Though the function of CO has not been clearly elucidated; in low concentrations, CO has antiapoptotic properties as well as vascular protective and anti-inflammatory properties,[54,55] which may contribute to blood pressure regulation. The effects of CO include vasodilation, inhibition of vascular smooth muscle cells proliferation, and induction of angiogenesis.[56,57] Accumulating evidence suggests that the HO pathway, through the production of CO and bilirubin, may be responsible for regulating vascular function as well as blood pressure.[39] Findings from the current study do not provide strong evidence for a causal association between circulating total bilirubin and hypertension. This suggests that the inverse associations demonstrated in the current and previous observational studies may be driven by biases such as unmeasured confounding and/or reverse causation.

Implications of Findings

Irrespective of the weak protective effective of total bilirubin on future hypertension risk and lack of evidence of a strong causal role, its potential role in the prevention and control of hypertension as well as CVD has been the subject of considerable debate.[14,18–20] Proven interventions that induce increase in safe levels of circulating bilirubin leading to clinically relevant decreases in blood pressure are currently unavailable. Lifestyle interventions (such as smoking cessation, weight loss, and physical activity)[58,59] and pharmacological agents (HO-1 inducers such as statins, aspirin, resveratrol, and niacin and drugs that inhibit hepatic uptake)[60,61] may cause safe elevations in circulating levels of bilirubin, but there is limited evidence on their potential effects on blood pressure. These pharmacological agents cause mild-to-moderate elevations in circulating levels of bilirubin and have been proposed as future tools for CVD prevention and treatment.[62] It has been suggested that the HO-1 pathway may represent a novel therapeutic target for the prevention of CVD.[39] The measurement of circulating total bilirubin involves a routinely available blood test, which is simple, cheap, and well standardized; therefore, it would be of immense clinical benefit if bilirubin is demonstrated to have a role in preventing hypertension as well as CVD. However, further research is needed and caution is required given that markedly elevated levels of bilirubin levels may exert toxic effects[4,63] and cause an increase in the risk of CVD[64,65] and mortality.[66]

Strengths and Limitations

The strengths of the current study include the large, population-based cohort, which was representative of the general population; exclusion of hypertensive individuals at baseline as well as those with pre-existing diseases, such as CVD, renal disease, or malignancy, which minimized any possibilities of reverse-causation bias; comprehensive data on lifestyle and biochemical factors, which allowed control for potential confounders; comprehensive analysis, such as evaluating the shape of the association and effect modification by clinically relevant characteristics; and robustness of the findings in several sensitivity analyses. Furthermore, we have utilized an MR approach using summarized large-scale published data to assess the associations of a specific genetic variant for circulating bilirubin with blood pressure. A number of limitations should also be considered when interpreting these results. First, because our data were observational, residual confounding attributed to errors in risk marker measurements and unmeasured confounders remains an alternative explanation. Second, absence of repeat measurements of total bilirubin precluded the ability to correct for within-person variability in plasma total bilirubin levels and this could have underestimated the associations, as a result of regression dilution given the long-term follow-up of the cohort.[67,68] Circulating bilirubin has been shown to exhibit high within-person variability,[69] hence the associations demonstrated may even be stronger. Larger-scale prospective studies with repeat measurements of circulating total bilirubin are needed to reliably assess the magnitude of the associations. Third, measurements of total bilirubin in the PREVEND study involved prolonged plasma storage, which could have contributed to the modest effect of total bilirubin on hypertension risk. Fourth, we assessed bilirubin concentrations in the fasting state, when the levels are highest. Because we had no data on nonfasting bilirubin concentrations, we were unable to investigate its concentrations in the nonfasting state and how these concentrations affect hypertension. Fifth, the current analysis involved principally white-European participants, which hampers the generalization of our findings. Finally, our MR approach was based on published publicly available data, which precluded the ability to fully assess instrumental variable assumptions. Given the unavailability of

interventions that specifically influence levels of bilirubin alone[70] and can be safely administered to large numbers of subjects for a prolonged period, causal inferences using randomized trials of interventions that modify bilirubin levels are not feasible in the short term. Further MR studies using large-scale, individual-level data may help to establish or rule out causality.

Conclusions

The weak and inverse association of circulating total bilirubin with future hypertension risk may be driven by biases such as unmeasured confounding and/or reverse causation. However, given the limitations of the present study, further evaluation is needed to rule out any causal association and assess any potential relevance of circulating total bilirubin in the prevention of hypertension.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. STROBE 2007 Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Page 1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 3
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 3-4
Methods			
Study design	4	Present key elements of study design early in the paper	Study population
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Study population
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Study population
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Risk Factor Assessment
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Risk Factor Assessment
Bias	9	Describe any efforts to address potential sources of bias	Statistical Methods
Study size	10	Explain how the study size was arrived at	Statistical Methods
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Statistical Methods

Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Statistical Methods
		(*b*) Describe any methods used to examine subgroups and interactions	Statistical Analyses
		(*c*) Explain how missing data were addressed	Not applicable
		(*d*) If applicable, explain how loss to follow-up was addressed	Not applicable
		(*e*) Describe any sensitivity analyses	Statistical Methods
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Study population
		(b) Give reasons for non-participation at each stage	Study population
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Results; Tables 1 and 2
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	Results
Outcome data	15*	Report numbers of outcome events or summary measures over time	Results
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results; Table 3
		(*b*) Report category boundaries when continuous variables were categorized	Results; Table 3
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Results; Figure 2; Tables S2 and S3
Discussion			
Key results	18	Summarise key results with reference to study objectives	Discussion - Summary of main findings

Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion
Generalisability	21	Discuss the generalisability (external validity) of the study results	Discussion
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Page 16

Table S2. Association of baseline total bilirubin levels with incident hypertension in several sensitivity analyses.

	Events/ Total	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Exclusion of first 2 years of follow-up	1,112 / 3,895	0.86 (0.81 to 0.91)	< 0.001	0.93 (0.87 to 0.99)	0.030	0.93 (0.87 to 0.99)	0.040	0.94 (0.88 to 1.00)	0.061
Exclusion of people with diabetes at baseline	1,196 / 3,970	0.87 (0.82 to 0.92)	< 0.001	0.94 (0.88 to 0.99)	0.037	0.94 (0.88 to 0.99)	0.040	0.94 (0.88 to 1.00)	0.066
Exclusion of people on cholesterol lowering medication	1,163 / 3,892	0.86 (0.81 to 0.91)	< 0.001	0.93 (0.87 to 0.99)	0.018	0.92 (0.87 to 0.99)	0.017	0.93 (0.87 to 0.99)	0.032
Exclusion of people with potential Gilbert's disease	1,205 / 3,985	0.86 (0.81 to 0.92)	< 0.001	0.93 (0.88 to 0.99)	0.032	0.94 (0.88 to 0.99)	0.037	0.94 (0.88 to 1.00)	0.063

CI, confidence interval; HR, hazard ratio; Q, quartile; SD, standard deviation; HRs are estimated per 1 SD increase in $\log_e$ total bilirubin

Model 1: Age and sex
Model 2: Model 1 plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation)
Model 3: Model 2 plus $\log_e$ urinary albumin excretion and $\log_e$ homeostasis model assessment of insulin resistance

Model 4: Model 3 plus $\log_e$ high-sensitivity C-reactive protein

Table S3. Association of baseline total bilirubin levels with incident hypertension using complex survey analyses.

Serum total bilirubin level (µmol/l)	Events/ Total	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Per 1 SD increase	1,206 / 3,989	0.84 (0.79 to	< 0.001	0.90 (0.84 to	0.004	0.90 (0.84 to 0.97)	0.005	0.91 (0.84 to 0.97)	0.007
Q1 (0.95-5)	367 / 1,134	ref		ref		ref		ref	
Q2 (6-7)	381 / 1,184	0.90 (0.78 to	0.186	0.97 (0.82 to	0.705	0.95 (0.81 to 1.13)	0.581	0.96 (0.81 to 1.13)	0.609
Q3 (8-9)	243 / 826	0.82 (0.69 to	0.018	0.94 (0.78 to	0.479	0.94 (0.79 to 1.13)	0.522	0.95 (0.79 to 1.14)	0.558
Q4 (≥ 10)	215 / 845	0.62 (0.52 to	< 0.001	0.75 (0.62 to	0.003	0.75 (0.61 to 0.91)	0.004	0.75 (0.62 to 0.92)	0.005

CI, confidence interval; HR, hazard ratio; Q, quartile; SD, standard deviation

Model 1: Age and sex
Model 2: Model 1 plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation)
Model 3: Model 2 plus $\log_e$ urinary albumin excretion and $\log_e$ homeostasis model assessment of insulin resistance

Model 4: Model 3 plus $\log_e$ high-sensitivity C-reactive protein

Figure S1. Frequency distribution of plasma total bilirubin in the study sample.

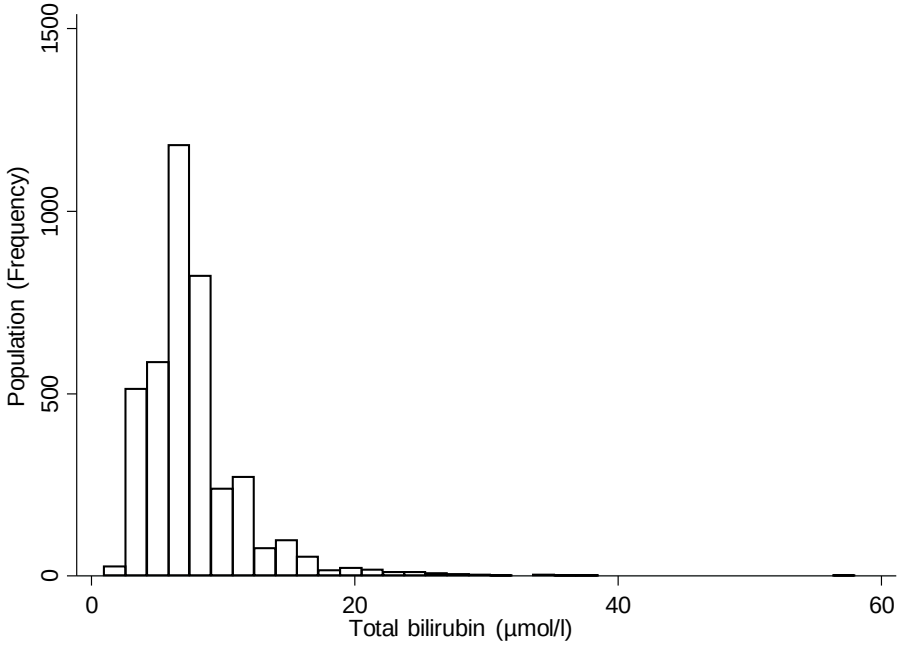
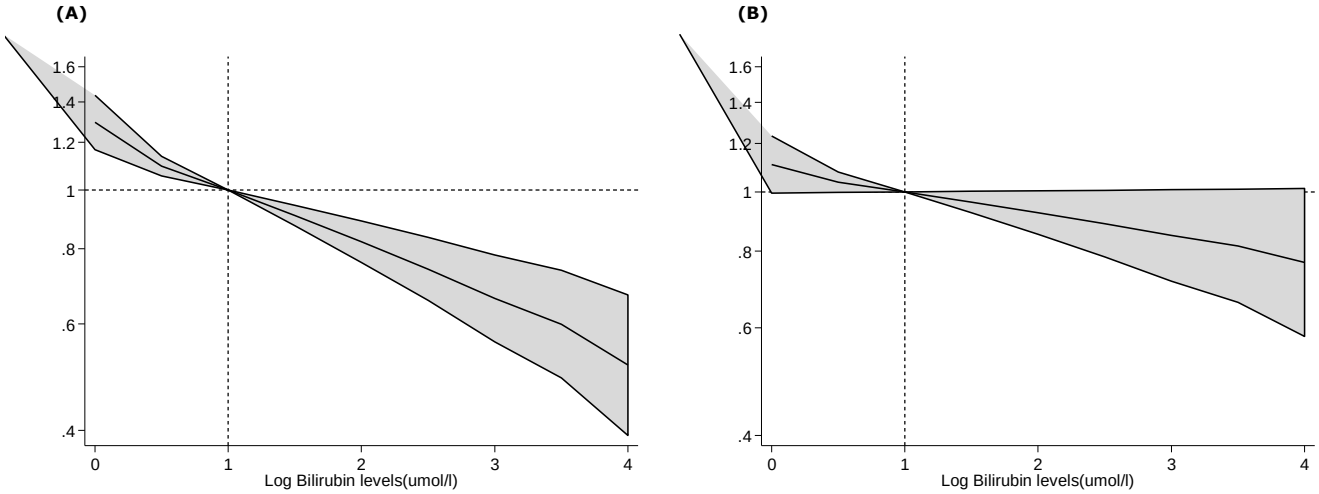


Figure S2. Hazard ratios for incident hypertension using multivariate-adjusted fractional polynomials.



A, Hazard ratios were adjusted for age and sex; **B**, adjustment in A plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, body mass index, parental history of hypertension, alcohol consumption, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation)

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